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Protein-Protein Interactive Networks Identified in Bronchoalveolar Lavage of Severe Compared to Nonsevere Asthma

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Abstract

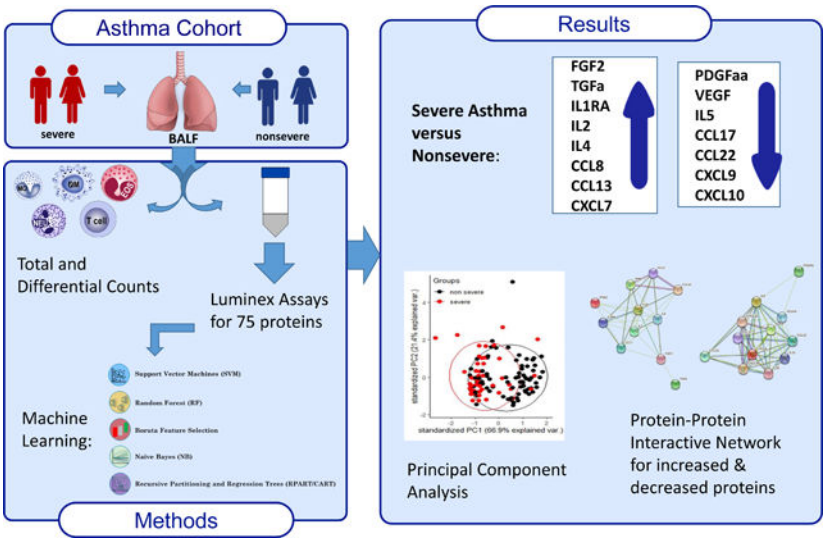
Introduction: Previous bronchoalveolar lavage fluid (BALF) proteomic analysis has evaluated limited numbers of subjects for only a few proteins of interest, which may differ between asthma and normal controls. Our objective was to examine a more comprehensive inflammatory biomarker panel in quantitative proteomic analysis for a large asthma cohort to identify molecular phenotypes distinguishing severe from nonsevere asthma.

Methods: BALF from 48 severe, and 77 nonsevere adult asthma subjects were assessed for 75 inflammatory proteins, normalized to BALF total protein concentration. Validation of BALF differences was sought through equivalent protein analysis of autologous sputum. Subjects' data, stratified by asthma severity, was analyzed by standard statistical tests, principal component analysis and 5 machine learning algorithms.

Results: The severe group had lower lung function and greater health care utilization. Significantly increased BALF proteins for severe asthma compared to nonsevere asthma were FGF2, TGFα, IL1Ra, IL2, IL4, CCL8, CCL13 and CXCL7; and significantly decreased were PDGFaa, VEGF, IL5, CCL17, CCL22, CXCL9 and CXCL10. Four protein differences were replicated in sputum. FGF2, PDGFaa and CXCL7 were independently identified by 5 machine learning algorithms as the most important variables for discriminating severe and nonsevere asthma. Increased and decreased proteins identified for the severe cluster showed significant protein-protein interactions for chemokine and cytokine signaling, growth factor activity, eosinophil and neutrophil chemotaxis differing between subjects with severe and nonsevere asthma.

Conclusion: These inflammatory protein results confirm altered airway remodeling and cytokine/chemokine activity recruiting leukocytes into the airways of severe compared to nonsevere asthma as important processes even in stable status.

Graphical Abstract



Keywords

severe asthma; bronchoalveolar lavage fluid; chemokines; cytokines; growth factors; machine learning

INTRODUCTION

Cellular gene expression from asthma BALF identified enriched components responding to cAMP signaling, potentially induced by immediately prior β-agonist use, but ELISA for these specific proteins in cell lysates did not show good agreement [1]. Those results confirm previously noted lack of linear association of gene expression with actual protein synthesized [2]. Thus, proteomic analyses may provide a more accurate view of the inflammation present in the lower airways.

Many reports have examined molecular differences in BALF proteins between normal subjects and patients with asthma [3–5], but usually were limited to small numbers of subjects or a few molecular mediators of interest. The results from these reports indicate increased inflammatory mediators in stable asthma or following allergen challenge, but provide little understanding of protein groups or possible molecular interactions between these. Recognition of heterogeneity in asthma [6] suggests that distinct patterns of cytokines, particularly associated with severe asthma, may be important, and call into question whether single biomarkers can distinguish between molecular endotypes. Limited numbers of cytokines and of subjects assessed may prevent more precise identification of increased or decreased inflammatory pathways associated with severe asthma phenotype or subgroups.

Our objective in this report was to investigate airway proteomic phenotype in a large cohort of adult subjects with a broad range of asthma severity assessed by a large inflammatory protein panel to elucidate patterns of protein heterogeneity between severe and nonsevere asthma. In addition, we examined replication in sputum samples, protein association with migratory leukocytes, and potential interactions between BALF proteins identified with

a severe asthma phenotype. A subset of the subjects and bronchoscopy data have been previously analyzed in reports that do not overlap with the results reported here [7–9]; the results in this report were presented as a conference abstract [10].

METHODS

Subjects:

77 nonsevere, 48 severe subjects from the National Heart Lung Blood Institute (NHLBI) Severe Asthma Research Program (SARP) 7 clinical sites, met American Thoracic Society (ATS) criteria for asthma and were classified by European Respiratory Society (ERS)/ATS guidelines [11]. Subjects who received high dose inhaled corticosteroid (≥880 mcg fluticasone equivalents per day) for at least 6 of the previous 12 months and 3 months prior to enrollment were assigned to the severe asthma group; those subjects with asthma not meeting these criteria were assigned to the nonsevere group [1]. Subjects signed local institutional review boards' (IRB) approved informed consent form and were comprehensively characterized (details in supplement) by IRB approved protocols at baseline. Subjects undergoing bronchoscopy showed similar differences between severe and nonsevere groups as the full cohort, but less severe if eligible for bronchoscopy; exclusion criteria: FEV1<40% predicted after 2.5 mg nebulized albuterol, or comorbidities of uncontrolled diabetes, uncontrolled coronary artery disease or hypertension. Bronchoscopy was performed only during a stable period for the subject (details in supplement) from April 2004 to December 2014. A subset of these subjects (N=48) had sputum supernatant samples with protein analysis available. All de-identified participant data will be deposited in dbGaP, and can be requested for legitimate scientific purposes through established channels (https://dbgap.ncbi.nlm.nih.gov/[dbgap.ncbi.nlm.nih.gov]). Additional related documents (protocol, methods of procedure [MOP], and data dictionaries) may also be made available upon request.

BALF processing:

Pooled return, from 100ml normal saline instilled, was centrifuged to separate cells and fluid. BALF aliquots were stored at −80°C; the cell pellet resuspended in 2ml of phosphate buffered saline, for total cell number and differential leukocyte percentages determinations at each clinical site [7,8]. Differential leukocyte percentages were multiplied by total cell count/ml obtained for cell pellet suspension. BALF was concentrated either 10 or 20X; total protein concentration was determined by enhanced Pierce BCA protein assay. Equal volume of each concentrated BALF sample was assessed (75 inflammatory mediators: Milliplex Human Cytokine/Chemokine assay Panels I, II, and III; Millipore Sigma); standards for linear curve determination included two control samples representing high and low levels for each target protein in the assay [12]. Specific protein levels were normalized to BALF total protein concentration.

Induced Sputum Processing:

Sputum samples were available from a subset (N=48) of the subjects with BALF protein analysis; processing and analyses of the sputum samples has been described [13, 14].

Sputum supernates were assessed by the Milliplex Human Cytokine/Chemokine assay Panels I, II, and III as performed for BALF.

Analyses and Statistics:

Subjects' demographic, clinical characteristics and inflammatory mediator levels for severe and nonsevere groups were analyzed by standard parametric and non-parametric tests for continuous or categorical values (SigmaPlot, ver 14.5, San Jose, CA). Multiple linear regression models were adjusted for asthma severity, age, gender, inhaled corticosteroid (ICS) use, baseline FEV_1%predicted, and BALF eosinophils. Principal Component Analysis (PCA) and five different machine learning (ML) algorithms were applied to the 52 cytokines/chemokines/growth factors with >50% of subjects having determinations above the lower limit of detection. Individuals with a missing value for any of these 52 proteins had one-half the lowest value observed for that protein substituted in ML algorithms. R statistical software (version 4.0.3) was used to build the ML models [15]. PCA positive or negative subgroups of severe and nonsevere subjects were examined for clinical and protein concentration differences. Inflammatory proteins found to differ between severe and nonsevere groups, or PCA positive and negative subgroups, were examined in String_db.org (version 11.5 [16]) for protein-protein interactions (PPI), functional enrichments of biological process and molecular functions, with False Discovery Rates (FDR) of $<e^{-04}$ reported.

RESULTS:

Subject Characteristics:

Demographics and clinical characteristics of subjects with severe or nonsevere asthma enrolled in the bronchoscopy portion of the SARP program and with BALF proteomic analysis are presented in Table 1. Severe and nonsevere groups differed for age, asthma duration, FEV1% predicted and FVC% predicted, both pre- and post-bronchodilator, controlling medications, emergency visits or hospitalizations for breathing problems. Although the % return of lavage fluid was lower in the severe asthma subjects (additional information for % return BALF is provided in the supplement), there were no differences in total cell count, total protein content, or specific leukocyte percentages in either the BALF or in a recent sputum sample.

Inflammatory Molecular Characteristics:

Of the 75 specific proteins in BALF assessed, 23 had fewer than 50% of subjects with detectable levels (online supplement Table S1 and additional supporting information). Analyses of these 23 proteins found no significant difference between severe and nonsevere asthma therefore, these proteins were excluded from further investigation. The median number of subjects having a missing level for any of the remaining 52 proteins was 11 (Q1-Q3, 2.5–31.5) or less than 10% of subjects. Univariate analysis of these 52 cytokines, chemokines, and growth factors identified 8 increased and 7 decreased in subjects with severe asthma compared to nonsevere asthma (Table 2). Removal of 5 severe asthma subjects who reported omalizumab medication at baseline from the analyses resulted only in

a loss of significance for BALF IL5 levels in severe subjects compared to nonsevere subjects (severe IL5=0.72pg/mg; nonsevere IL5=1.14 pg/mg; p=0.066).

The 15 inflammatory proteins, increased or decreased in BALF univariate analyses in the severe asthma group, were examined in String-db.org [16] and formed recognized linkage groups (see additional results in supplement and Figure S1). The remaining 37 proteins examined showed no statistical difference between severe and nonsevere asthma subjects (online supplement Table S1). Multivariate analysis adjusting for asthma severity, age, sex, ICS use, baseline FEV_1 %predicted, and BALF eosinophil count resulted in 4 proteins remaining significant for severe compared to nonsevere subjects: increased CCL13/MCP4 and CXCL7/NAP2; decreased PDGFaa and VEGFa.

Available protein analysis of autologous sputum supernatants were examined for validation of observed specific BALF proteins' differences between severe compared to nonsevere subjects. Of the 75 proteins examined in sputum, only 12 had fewer than 50% of subjects with detectable levels; 10 of these sputum components were identical to BALF proteins with <50% of subjects with detectable levels (Supplement Table S1). Four of the 15 BALF proteins which differed between severe and nonsevere groups were significantly different in sputum as well: IL1Ra and IL4, both increased in severe subjects, and CXCL9/MIG and CXCL10/IP10, both decreased in severe subjects (Table 2).

Cell-Protein Associations:

Multiple variable linear regression models with specific BALF leukocyte counts/ml showed significant positive or negative associations for 33 of the 52 proteins (R values >0.32 to <0.67, p values <0.02). These were predominantly positive associations with BALF cell counts, but varied with respect to specific leukocyte (online supplement Table S2). Nine of the initial 15 proteins observed to differ between severe and nonsevere subjects (TGFa, IL2, IL5, CCL8, CCL17, CCL22, CXCL9, CXCL10 and VEGFa) had significant associations with BALF leukocytes, but individually were associated with different specific leukocytes. Positive associations (N=18 of the 33 proteins with BAL cell associations) were primarily with lymphocytes; only 2 were negative. Eosinophils had significant positive associations with 10 proteins; macrophages/monocytes had eight positive inflammatory proteins associations, but were negatively associated with IL2. Neutrophil counts were only associated with 3 proteins in BALF.

Machine Learning Analysis:

All 52 biomarker proteins having at least >50% of subjects with detectable values were initially examined by PCA (Figure 1A). Subject clusters had considerable overlap and poorly explained variance. Therefore, machine learning algorithms were explored to determine the most important features among the 52 proteins. Application of five distinct algorithms reduced redundancy of selection, and taking the most commonly selected features is the most successful approach for feature selection [17]. The five different algorithms identified three proteins as key features: PDGFaa (all 5 algorithms), FGF2 (4 algorithms), and CXCL7/NAP2 (3 algorithms)(Table 3) which were examined by PCA (Figure 1B). This approach yielded greater area under the curve in receiver operating curve

analysis to predict severe asthma than use of all 52 proteins (Figure 1C). Adding the next 3 features identified by CART, NB and SVM algorithms (CCL17/TARC, CCL22/MDC, and IL-7) to the initial 3 proteins did not enhance partitioning between the severe and nonsevere groups (online supplement Figure S2) nor did these improve explained variance.

PCA performed with the top 3 identified proteins (Figure 1B) clustered the majority of severe subjects (37/48, 77%) according to PC1 with 66.9% explained variation. A somewhat lower percentage of nonsevere subjects clustered together (49/77, 64%) according to PC1. Based on the distribution along the PC1 axis with a threshold of "0" for that axis, the severe and nonsevere groups each split into 2 subgroups, either PC1 positive or negative.

Clinical characteristics for these 4 subgroups (Table 4) had similar results for age, lung function, medication use and BALF leukocyte differentials between severe and nonsevere subgroups, whether PC1 positive or negative, but BALF total cell count differed. BALF total cell count was double or greater in the PC1 'positive' subgroups, both severe and nonsevere, than in the PC1 'negative' subgroups, but this difference was not attributable to increased BALF yield, percentages of specific leukocytes, or medication differences between PC1 'positive' and 'negative' subgroups. (Additional information regarding protein adjustment for total cell count is provided in the supplement.)

Specific protein levels adjusted to total BALF protein concentration, re-examined across the 4 subgroups (severe PC1 negative or positive, and nonsevere PC1 positive or negative) resulted in additional increased and decreased proteins by one-way ANOVA. Those meeting significance for comparison of severe PC1 negative group to nonsevere PC1 positive group (post-hoc Dunn's test) are listed (online supplement Table S3) The additional increased proteins in the PC1 negative groups (severe and nonsevere) included IFNα2, CCL7/MCP3, and IL-10. Additional decreased proteins in the PC1 negative groups included CCL1/I309, CCL14a/HCC1, IL1β, IL6, IL16, and CXCL11/I-TAC.

Protein-Protein Interactions (PPI), Biological Process and Molecular Functions Identified:

PPI for significantly increased and decreased proteins for the PC1 negative subgroups were examined in String-db.org (Figure 2A [increased] and 2B [decreased]). The additional identified proteins resulted in enhanced PPI and p values for both increased and decreased groups: proteins increased in PC1 negative groups had 11 nodes with 33 edges, PPI enrichment p value $<1.0e^{-16}$; proteins decreased in PC1 negative groups had 12 nodes with 51 edges, PPI enrichment p value $<1.0e^{-16}$. Importantly, all increased and decreased groups of inflammatory mediators represented nodes connected in significant interactive networks.

Functional enrichments for biological processes reported in string-db.org for the increased proteins in PC1 negative groups were eosinophil chemotaxis (FDR $7.28e^{-05}$), neutrophil chemotaxis (FDR $4.40e^{-05}$), chemokine-mediated signaling pathway (FDR $5.45e^{-05}$) and positive regulation of receptor signaling pathway via jak-stat (FDR $5.97e^{-05}$). Molecular functions for increased proteins included growth factor activity (FDR $6.35e^{-08}$), growth factor receptor binding (FDR $2.96e^{-08}$), cytokine activity (FDR $2.54e^{-15}$), cytokine receptor binding (FDR $6.08e^{-13}$), and chemokine activity (FDR $8.22e^{-06}$).

Biological processes reported in String-db.org for decreased proteins in PC1 negative groups included lymphocyte chemotaxis (FDR $2.32e^{-10}$), monocyte chemotaxis (FDR $2.48e^{-8}$), granulocyte chemotaxis (FDR $4.25e^{-16}$), neutrophil chemotaxis (FDR $4.65e^{-14}$), myeloid leukocyte migration (FDR $4.15e^{-17}$), and chemokine-mediated signaling pathway (FDR $2.06e^{-11}$) among other processes. Molecular functions for decreased proteins included C-X-C chemokine receptor 3 (CXCR3) and C-C chemokine receptor (CCR) binding (FDR $2.89e^{-06}$ and FDR $5.37e^{-06}$, respectively), chemokine activity (FDR $3.93e^{-13}$) and cytokine activity (FDR $3.45e^{-17}$), and cytokine receptor binding (FDR $1.02e^{-14}$).

DISCUSSION:

A large panel of inflammatory proteins assessing BALF from a substantial cohort of subjects with asthma identified several molecular differences, both increased and decreased, between severe and nonsevere as defined by ERS/ATS guidelines [11]. CCL13/MCP4 and CXCL7/NAP2 remained significantly increased, and PDGFaa and VEGFa remained significantly decreased in the severe asthma group in analyses adjusted for potential confounders. Five machine learning algorithms separately identified 3 proteins, increased FGF2 and CXCL7/NAP2, and decreased PDGFaa, as the most important features. PCA with these 3 proteins defined 2 clusters; a PC1 ‘negative’ cluster contained 77% severe asthma subjects, and a PC1 ‘positive’ cluster contained 64% nonsevere asthma subjects. Additional proteins were observed significantly increased and decreased across these two clusters. PPI for all increased proteins were enhanced, highlighting functional and molecular enrichment for eosinophil and neutrophil chemotaxis, chemokine-mediated signaling, positive regulation of jak-stat signaling, growth factor receptor binding and activity. In contrast, PPI for significantly decreased proteins represented leukocytes’ chemotaxis, chemokine mediated signaling, CXCR3 and CCR receptor binding, chemokine and cytokine receptor binding and activity. These important observations define molecular pathways underlying severe asthma during stable status, and may support use of JAK inhibitors in those patients unresponsive to corticosteroid therapy or anti-Type 2 biologics [18]. Increased proteins’ recruiting both eosinophils and neutrophils in severe asthma airways, may be counterbalanced by decreased proteins regulating granulocyte chemotaxis. Thus, BALF granulocyte percentages did not differ between severe and nonsevere asthma groups. Nor did sputum cell percentages differ for the subset of subjects with autologous sputum data.

However, important functional details may differ despite similar differential leukocyte percentages in severe and nonsevere BALF. Differences in expression of receptors CXCR3 and CCR5 expression noted in BAL cells could alter response to CXCL9, CXCL10, and CXCL11 ligands for CXCR3, or CCL5 ligand for CCR5 [19]. Al-Rashoudi et al. similarly noted differential expression of CCR2 and CX3CR1 on CD16+ monocyte subsets associated with asthma severity [20]. Camiolo et al. [21] concluded separate molecular mechanisms contributing to two separate severe asthma groups, one enriched for IL4 positive cells. Our 2 PCA clusters defined by FGF2, CXCL7/NAP2 and PDGFaa show similar division of severe subjects. Moreover, decreased NK cells relative to $CD4^+$ T cells observed in severe asthma BAL [8], indicate different proportions of lymphocyte phenotypes, even though lymphocyte numbers may not differ.

Other airway cell types: innate lymphoid cells 2 (ILC2), airway epithelial cells, mast cells, dendritic cells and airway smooth muscle may all release various proteins into the airways [6]. Determining whether one or more of these cell types contributed some of the protein differences observed here was beyond the scope of this study, but is acknowledged.

Although our expanded analysis of 52 proteins out of 75 assessed in BALF from a large cohort of severe and nonsevere asthma patients confirms increased IL2 and IL4, and decreased CXCL9/MIG and CXCL10/IP10 reported by Brasier and colleagues [22–23], we found increased IL1Ra, contrasting to their decrease. Other BALF proteomic analyses reported for asthma are restricted to single inflammatory proteins of interest or differ in methodology; for example, high-sensitivity single protein ELISA [24] versus our multiplex Luminex assays, or a different proteomic assay platform such as Meso Scale Discovery [25]. Broad variation for correlations of specific protein between proteomics assays, such as SOMAscan, Meso Scale Discovery and Myriad Rules Based Medicine, shown in chronic obstructive pulmonary disease cohorts [26], reinforce caution in comparing results from different protein assays. Assessments of BALF proteins by mass spectrometry are generally limited in sample number analyzed; for example, Wu and colleagues [27] included only 4 patients with mild asthma compared to 3 healthy subjects. That approach excludes subgroup heterogeneity characterized by different molecular mechanisms within severe asthma, as observed here for the small number of severe subjects clustered with mainly nonsevere subjects in PCA results.

Complex overlapping patterns of types 1, 2 and 17 inflammatory mediators have been observed in both BAL [28] and sputum [13], which associate with increasing neutrophils in severe asthma [13, 14, 28, 29]. Although our observations of increased BALF IL2, IL4 and CXCL7 and decreased CXCL10 correspond with previous observations in sputum from severe asthma subjects [13], other proteins, significant in BALF, did not differ in sputum from autologous subjects, suggesting separate, but overlapping, lung compartments. Cell type differentials, and dissimilarities in protein associations with specific leukocytes in BALF and sputum further support distinct compartments. In fact, the majority of the BALF proteins significantly associate with lymphocyte counts, whereas, sputum proteins associate primarily with neutrophil counts [13]. Thus, comparison of SARP BALF proteins, for example with UBIOPRED sputum proteomic analyses [30, 31], may differ not only due to technical differences in proteomic assays [26] but also inherent sample differences.

BALF inflammatory protein results support complex protein interactions [13]. including significant interactions between Types 1 and 2 inflammation. The identification of protein-protein linkage groups containing Type 2 and other T cell regulators of inflammation emphasize heterogeneity underlying pathologic processes important in severe asthma [32, 33]. Growth factor enrichment points to airway remodeling, supported by observed elevated FGF2 in sputum of severe asthma, inversely correlating with FEV_1/FVC ratio [34]. VEGF is induced by IL-4,, and subsequently induces angiogenesis in asthmatic airways [35], but we found decreased VEGFa despite increased IL-4 in our severe subjects' BALF. Interestingly, increased CCL13/MCP4, recruits eosinophils in asthma [36,37], is released from A549 alveolar type II cells upon stimulation by IL4 [38], and along with CCL7 and CCL8, is located on chromosome 17q11, adjacent to a region strongly identified with

asthma susceptibility and severity [39]. These highlighted molecular mechanisms indicate alternative areas to explore for treatments for subjects less predominantly characterized by type 2 inflammation, resistant to corticosteroids, or unresponsive to anti-Type 2 biologics [40].

A limitation already mentioned for this study is the total number of proteins, N=75, which although larger than previously examined [22,23], is less than what may be evaluated by other methods. Levels below detection limits for 23 of the 75 proteins in BAL assessed was a further constraint, despite BALF concentration before assay. Thus, we were unable to further assess certain inflammatory proteins, including IFNγ, IL13, IL17, IL33, and TSLP among others, which have been associated with severe asthma [41–43], to determine whether those proteins' concentrations differed in this cohort.

Conclusion:

In summary, we have examined inflammatory proteins identified in BALF from subjects with severe and nonsevere asthma. Through PCA, machine learning algorithms, and string analysis the identified proteins defined 2 clusters of subjects with primarily severe or nonsevere asthma. Increased and decreased proteins differentiating the 2 clusters comprised significant PPI networks with functional enrichment for eosinophil and neutrophil chemotaxis, positive regulation of signaling pathways via jak-stat, and growth factor activity. These additional identified protein networks underlying pathologic inflammatory mechanisms in severe asthma suggest novel therapy targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

ATS	American Thoracic Society

BALF	bronchoalveolar lavage fluid
CART	classification and regression trees
CCL	C-C chemokine
CCR	C-C chemokine receptor
CXCL	C-X-C chemokine
CXCR	C-X-C chemokine receptor
ELISA	enzyme-linked immunosorbent assay
ERS	European Respiratory Society
FDR	False discovery rate
FGF2	fibroblast growth factor 2
ICS	inhaled corticosteroid
IL	Interleukin
ILC	innate lymphoid cells
IRB	Institutional Review Board
NB	naïve Bayes classifier
PCA	principal component analysis
PDGFaa	Platelet derived growth factor a-a dimer
PPI	protein-protein interaction
RF	random forest classification
SARP	Severe Asthma Research Program
NHLBI	National Heart Lung Blood Institute
SVM	support vector machine
TGFa	transforming growth factor a
VEGF	vascular endothelial growth factor

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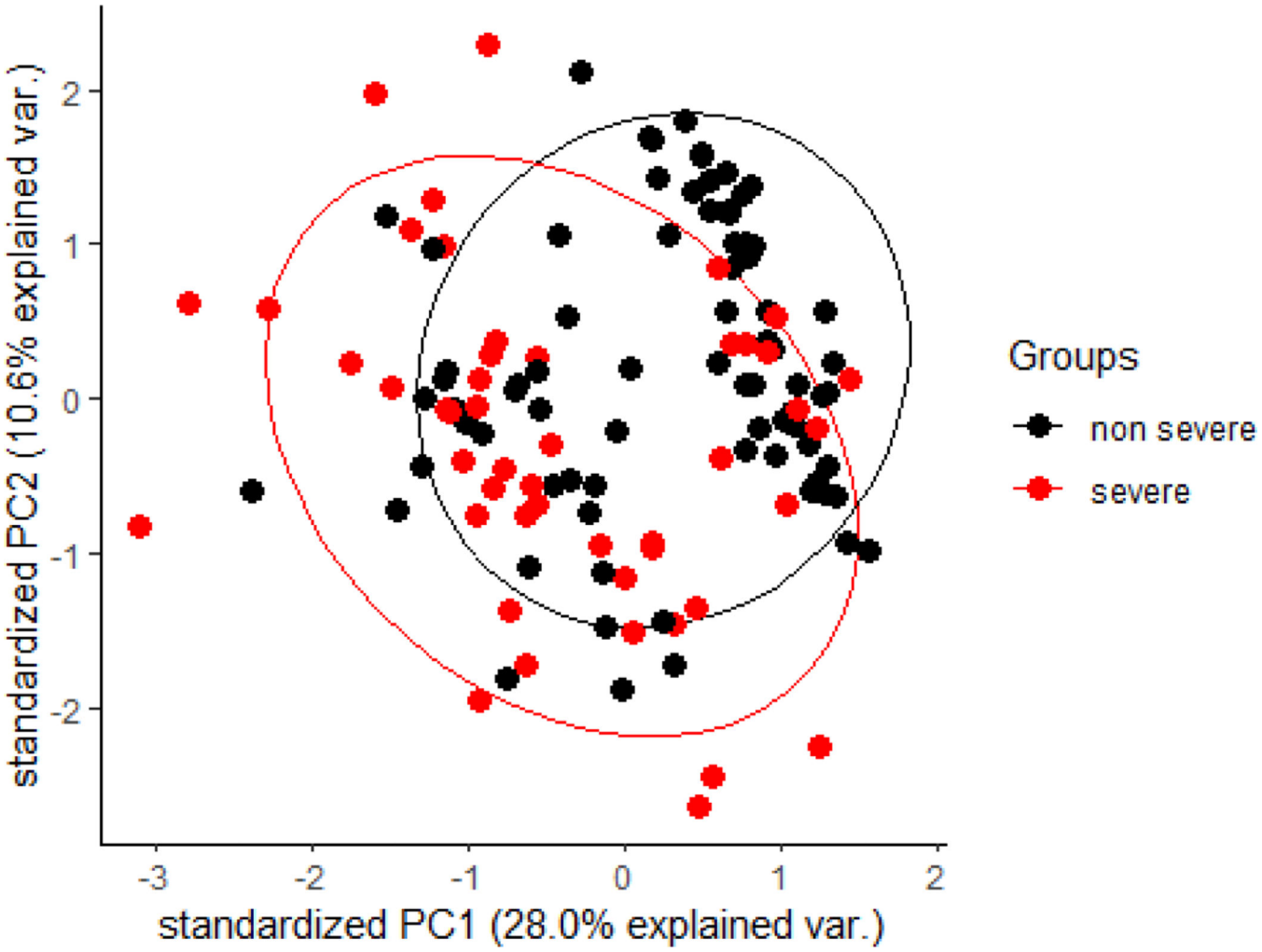
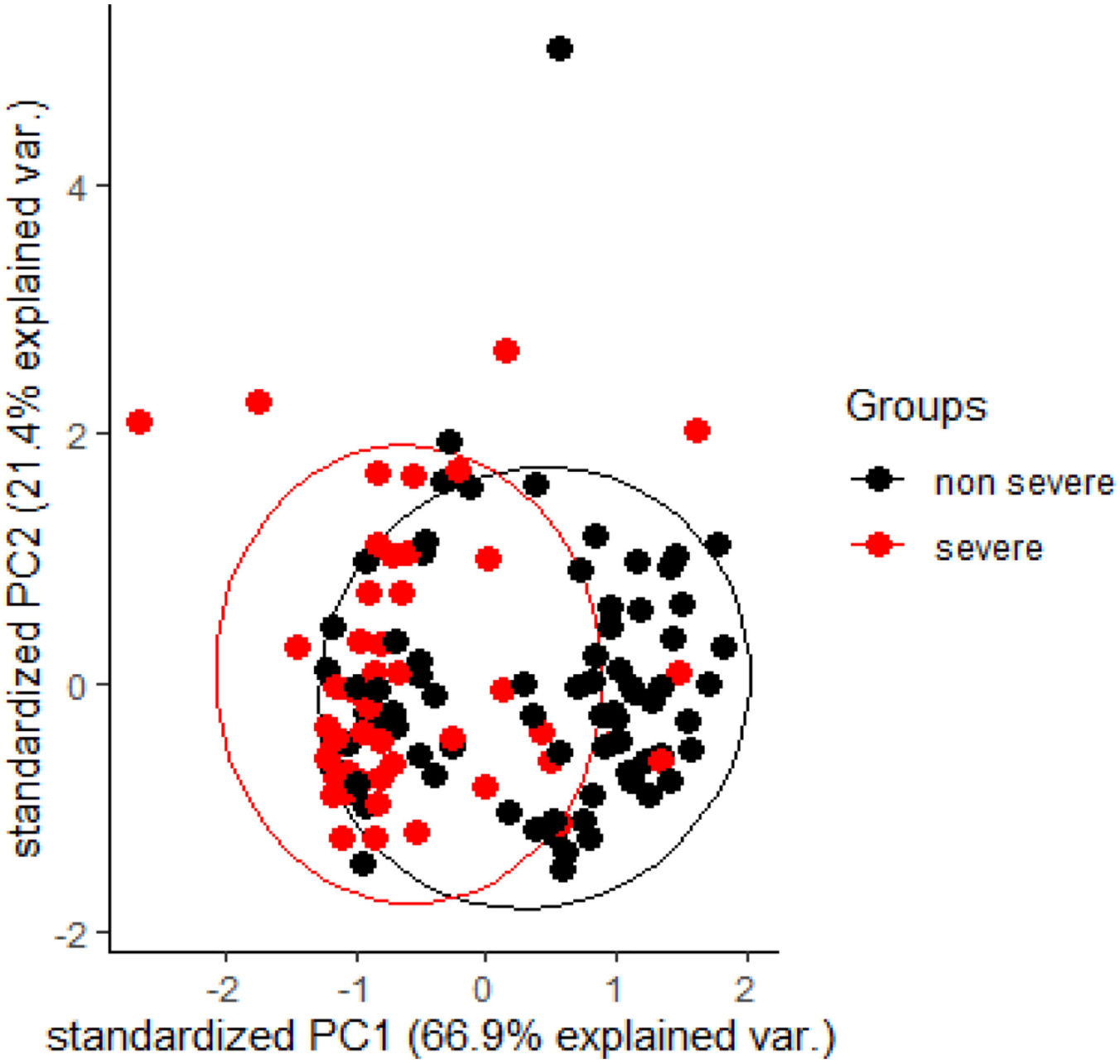
Key Messages:

- Proteomic analysis of BALF identified increased and decreased proteins which differentiate severe from nonsevere asthma.
- FGF2, CXCL7 and PDGFaa were key features differentiating two clusters, predominantly severe or nonsevere asthma.
- Significant protein-protein interactions identified confirm airway remodeling, receptor signaling, and leukocyte recruitment in severe asthma.





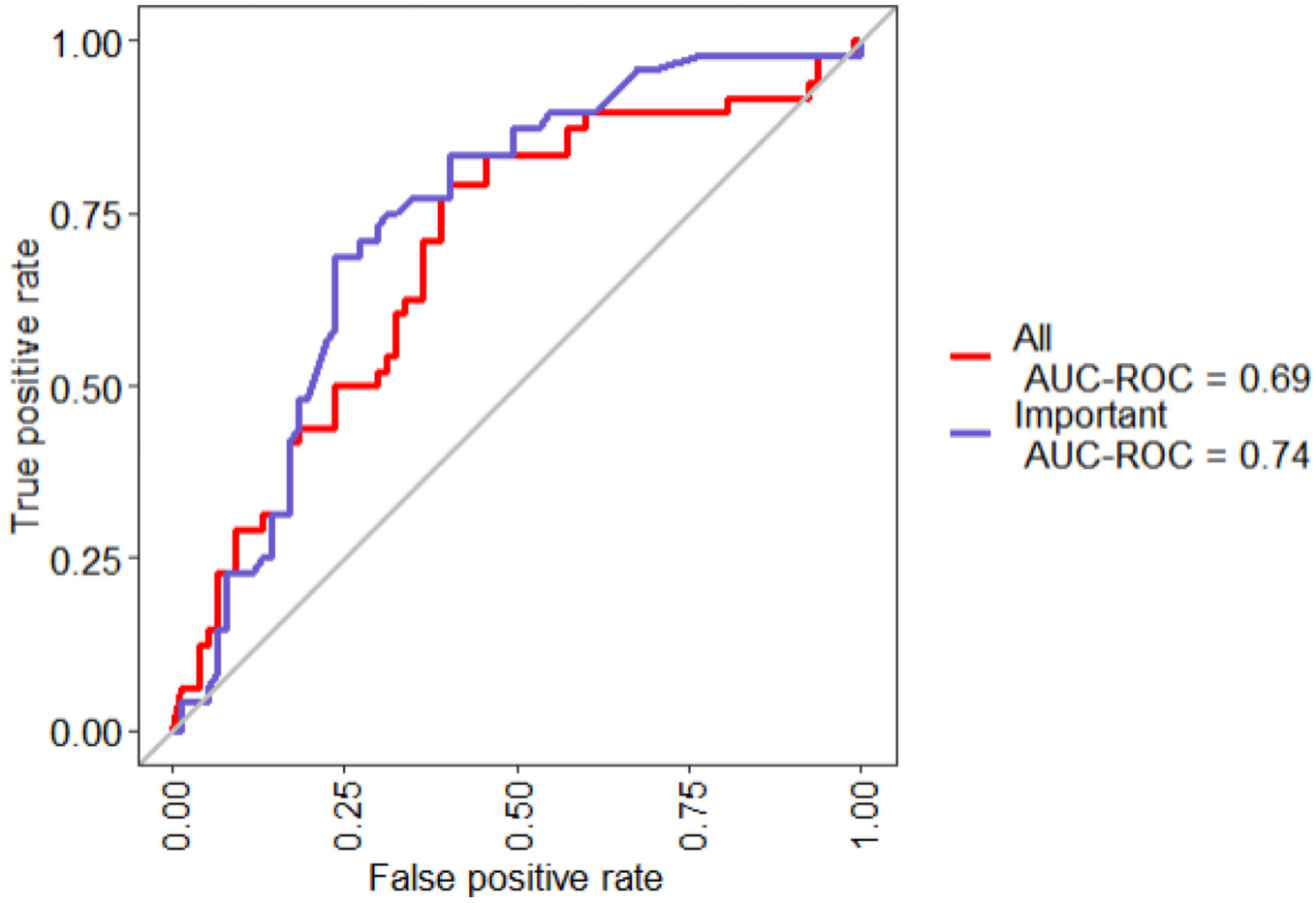
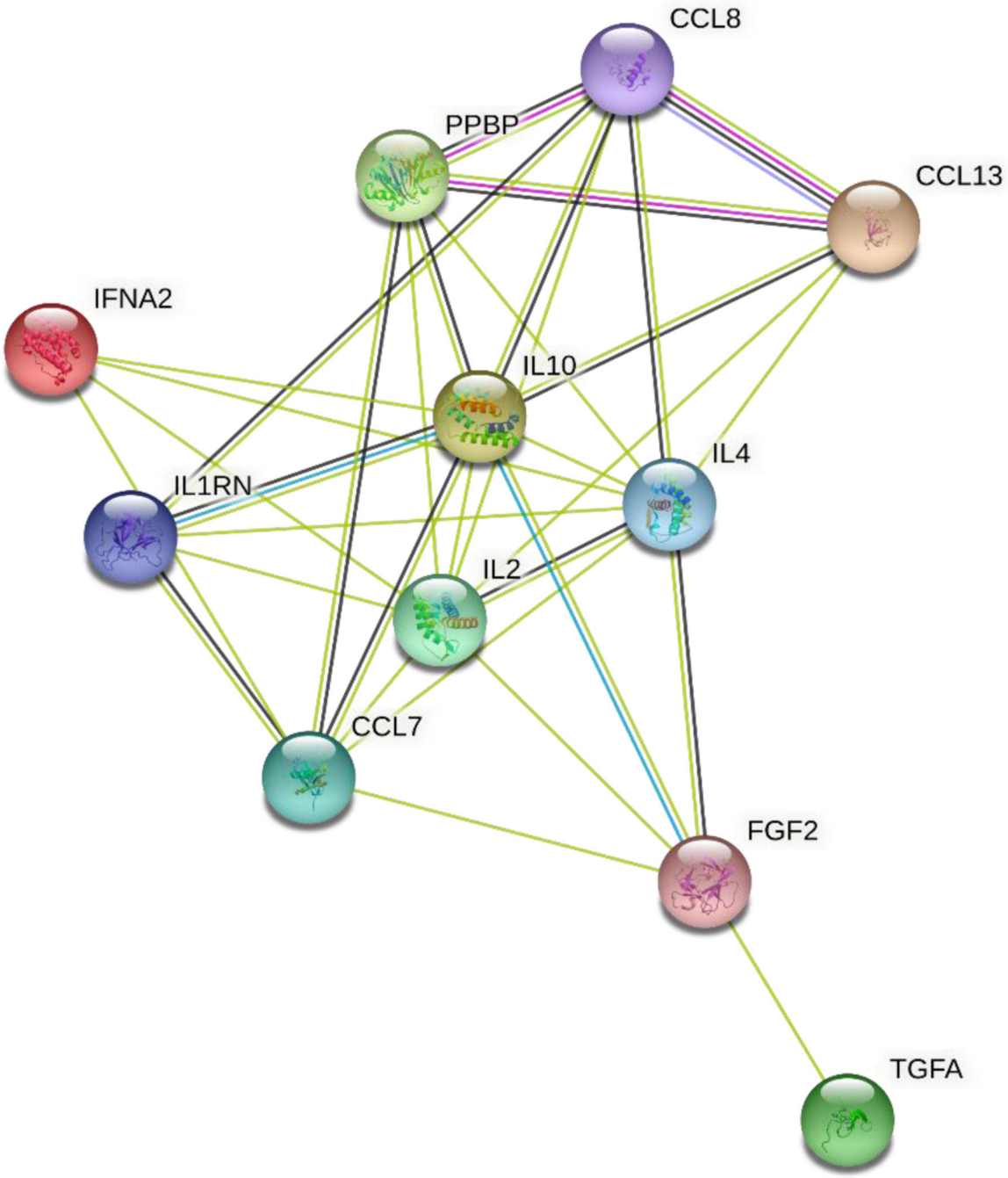


Figure 1:

A:PCA distribution of subjects according to analysis with 52 cytokines, chemokines and growth factors. Subjects classified as severe (red markers) or nonsevere (black markers) by ERS/ATS guidelines are indicated. The two clusters overlap with only 28% of PC1 explained variation. **B:** PCA distribution of subjects according to top 3 features (PDGFaa, FGF2 and CXCL7/NAP2) identified by machine learning. PCA 1 shows two main clusters, one primarily of severe and one mainly of nonsevere subjects, although some severe subjects group with nonsevere, and some nonsevere subjects group with severe. The explained variation for PC1 in this analysis is more than double at 66.9%. Ellipses in both PCA figures indicate the 95% confidence limits. **C:** Comparison of Receiver Operating Curves (ROC) for all 52 proteins (red line) and for top three features (blue line). The top three features have a better area under the curve (AUC=0.74) for predicting severe subjects than all 52 proteins (AUC=0.69).



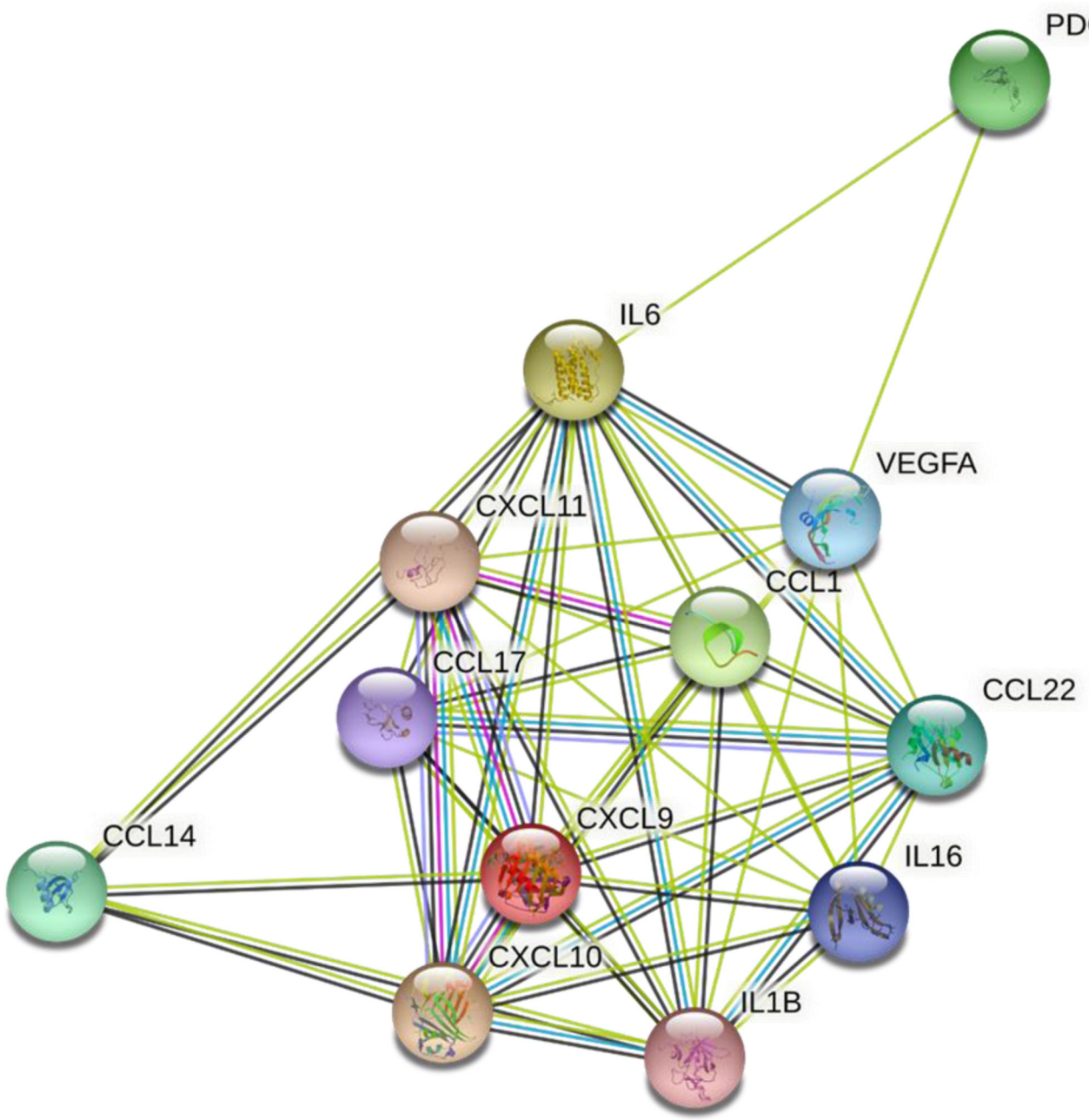


Figure 2:

A:Protein-Protein Interactions between increased biomarkers identified by PC1 ‘negative’ subjects, both severe and nonsevere. The node designated as PPBP is CXCL7. **B.** Protein-Protein Interactions between decreased biomarkers identified for PC1 ‘positive’ subjects, both severe and nonsevere.

Table 1.

Subject demographics and clinical characteristics with comparison of severe to nonsevere asthma subgroups. Levels given as mean +/− standard deviation from t-test, or median (25%−75% intraquartile) from Mann-Whitney (if not meeting test for normal distribution), or % positive for the group as indicated for each variable.

Variables:	Nonsevere (N=77)	Severe (N=48)	P-value
Age	33.57 (24.84–43.6)	45.86(33.31–52.96)	**<0.001** *
Sex (% Female)	69.74	60.42	0.38
Race (%White/%AA/%Other)	57.9/38.2/3.9	54.2/39.6/6.3	0.82
Asthma Duration	22.07 +/− 10.62	27.64 +/− 12.94	**0.018** *
Body Mass Index	28.17 (23.76–34.02)	29.59 (25.87–36.38)	0.16
Baseline FEV_1%predicted	82.17 +/− 13.99	70.95 +/− 19.00	**<0.001** *
Baseline FVC%predicted	93.82 +/− 13.93	84.93 +/− 17.07	**0.003** *
FEV_1/FVC	0.72 (0.69–0.76)	0.71 (0.68–0.73)	0.20
Maximum FEV_1%predicted	93.73 +/− 14.12	84.40 +/− 18.97	**0.005** *
Maximum FVC%predicted	99.79 +/− 13.75	93.24 +/− 16.66	**0.026** *
PC_{20}	0.94 (0.35–2.26)	0.62 (0.29–0.93)	0.09
IgE	159.0 (90.50–34)	172.2 (83.70–528.80)	0.60
Number of positive allergen tests	5 (3–8)	6.5 (2–9)	0.80
Log FeNO (ppb)	1.54 +/− 0.33	1.39 +/− 0.33	**0.019** *
Total % positive for ICS	66.67	100	†
Total % positive for LABA	46.05	93.75	†
Oral Corticosteroid (%)	0	16.67	†
Leukotriene receptor antagonist (% positive)	13.16	37.5	†
Emergency Visit for breathing problem in past year (% positive)	12.86	45.83	†
Intubation For Breathing problem ever (% positive)	4.29	16.67	†
Hospitalization for Breathing problem ever (% positive)	40.00	60.42	†
BALF Yield (% return)	51.3±14.3	38.3±14.1	**<0.001** *
BALF Total Protein (concentrate)	1.06 (0.78–1.36)	0.95 (0.75–1.23)	0.30
BALF Total Leukocyte Count $x10^6$	6.7 (3.13–13.1)	4.9 (2.80–7.70)	0.09
BALF Macrophage%	92 (87.08–95.48)	91.25 (83.38–94.80)	0.21
BALF Lymphocyte%	5.7 (2.63–8.88)	4.8 (2.90–10.60)	0.83
BALF Neutrophil%	1.25 (0.50–2.30)	1.7 (0.38–3.93)	0.33
BALF Eosinophil%	0.3 (0–1.08)	0.35 (0–0.93)	0.84
Autologous Subjects with Sputum proteomics N	28	20	
Sputum Supernate Total Protein concentration mg/ml	2.10 (1.61–3.63)	2.77 (1.92–3.62)	0.34
Sputum Total Cell Count $x10^6$	1.6 (1–2.6)	1.45 (1–2.2)	0.82
Sputum %White Blood Cells	53.5 (30–79)	40.7 (15–71)	0.13
Sputum Macrophage%	43.7 (24–62)	30.9 (18–61)	0.13

Variables:	Nonsevere (N=77)	Severe (N=48)	P-value
Sputum Lymphocyte%	1.5 (1–3)	1.1 (0.4–2)	0.11
Sputum Neutrophil%	48.4 + 26.4	54.3 + 25.4	0.29
Sputum Eosinophil%	1.2 (0.2–4)	0.7 (0.2–2.5)	0.77

[*] P values considered significant are in bold font.

[†] These characteristics are criteria for “severe” asthma definition, and therefore differ, by definition, between severe and nonsevere asthma groups.

Table 2.

Cytokines, chemokines and growth factors differing in BALF between nonsevere and severe subjects with asthma. Univariate analysis for difference between severe and nonsevere subjects performed by T-test (or Mann-Whitney rank sum test if Shapiro-Wilk test for normality failed). Multivariate linear regression model was adjusted for variables: severe or nonsevere asthma classification, age, gender, use of inhaled corticosteroid, baseline FEV1%predicted, and BAL eosinophil count; p value given for the variable severe or nonsevere asthma category. Levels given are median value (25% −75% intraquartile) for groups for each protein which is presented as pg per mg of total protein.

***Increased Proteins* in Severe Asthma**	**Nonsevere subjects' BALF concentration (N=77)**	**Severe subjects' BALF concentration (N=48)**	**Univariate P value**	**Multivariate P value**	**Nonsevere Sputum concentration (N=28)**	**Severe Sputum concentration (N=20)**	**Sputum P value**
FGF2	30.01 (9.3–52.9)	43.02 (26.2–63.3)	**0.011** *	0.696	29.2 (17.6–43.0)	24.1 (21.1–28.6)	0.40
TGFa	3.48 (2.46–10.81)	3.97 (3.06–6.62)	**0.034** *	0.338	2.9 (2.0–6.5)	3.4 (1.2–7.4)	0.95
IL1Ra	96.78 (41.45–208.83)	175.18 (96.03–421-45)	**0.003** *	0.071	2146 (1523–6160)	5103 (2472–12.174)	**0.05** *
IL2	0.93 (0.43–1.57)	1.51 (1.07–1.94)	**0.002** *	0.457	0.68 (0.45–1.56)	0.62 (0.36–0.97)	0.42
IL4	7.60 (1.62–11.22)	11.09 (4.44–19.07)	**0.040** *	0.758	10.5 (1.8–43)	27.7 (10.1–62.5)	**0.04** *
CCL8/MCP2	5.76 (4.00–8.70)	8.27 (6.05–12.59)	**0.002** *	0.406	3.9 (3.5–5.1)	4.0 (3.5–4.6)	0.84
CCL13/MCP4	10.82 (7.84–17.38)	19.80 (10.45–30.34)	**0.003** *	**0.014** *	9.4 (7.2–17.8)	8.8 (6.5–15.4)	0.71
CXCL7/NAP2	714.3 (264.1–2421)	3735 (1329–6268)	**<0.001** *	**0.011** *	46.8 (26.7–77.7)	40.2 (11.9–59.3)	0.44
Decreased Proteins in Severe Asthma:							
PDGFaa	49.78 (16.85–95.66)	14.39 (7.94–25.73)	**<0.001** *	**<0.001** *	13.9 (2.5–101)	3.7 (1.8–76)	0.32
VEGFa	287.1 (110.8–464.3)	95.77 (53.62–227.7)	**<0.001** *	**0.038** *	82 (34–153)	51 (18–108)	0.10
IL5	1.14 (0.59–1.83)	0.51 (0.26–1.36)	**0.022** *	0.992	1.6 (0.9–3.6)	1.4 (0.9–2.3)	0.88
CCL17 (TARC)	6.74 (2.82–15.92)	3.11 (2.05–6.06)	**0.005** *	0.674	1.6 (0.8–3.4)	1.4 (0.7–5.7)	0.72
CCL22 (MDC)	86.36 (38.75–142,23)	28.24 (17.91–81.93)	**<0.001** *	0.125	13.7 (11.2–27.7)	10.9 (5.9–24.5)	0.34
CXCL9 (MIG)	585.8 (203.4–1364)	161.9 (77.7–950.5)	**0.014** *	0.745	3595 (847–10,855)	937 (184–2444)	**0.01** *
CXCL10 (IP10)	696.6 (330.2–1248)	316.8 (118.3–759.1)	**0.002** *	0.366	1559 (596–3634)	772 (128–1555)	**0.04** *

* P values considered significant are in bold font.

Table 3.

Specific BALF proteins (adjusted to total mg protein concentration of BALF) identified by X as most important features by the five different machine learning algorithms. The protein 'features' identified as the top 3 are highlighted by bold font; importance according to each algorithm is provided in parentheses.

Algorithm:	**Boruta feature selection**	**CART Modeling via rpart**	**Naive Bayes classifier**	**Support Vector Machine**	**Random Forest Classification**
Top Protein features:					
PDGF-AA	**X (10)**	**X (100)**	**X (100)**	**X (100)**	**X (1.5)**
FGF2	**X (4.5)**	**X (62)**	**X (67)**	**X (67)**	
CXCL7(NAP2)	**X (6)**		**X (56)**	**X (56)**	
IL-8		X			
CCL17/TARC		X (66)	X (70)	X (71)	
CCL22/MDC		X (60)	X (71)	X (71)	
IL-7		X (58)	X (69)	X (69)	
IFNalpha2		X			
sCD40L			X	X	
VEGF			X	X	
IL-4			X	X	
IL-2			X		X

Table 4.

Severe and Nonsevere groups split into subgroups based on PC1 distribution for PCA determined with FGF2, CXCL7 and PDGFaa. Levels given as mean +/− standard deviation from ANOVA, or median (25%−75% intraquartile values) from Kruskal Wallis (if not meeting test for normal distribution), or % from Chi-square as indicated.

Variable	Nonsevere PC1 positive	Nonsevere PC1 negative	Severe PC1 positive	Severe PC1 negative	P value
N	49	28	11	37	
Age in years	33.3 (24.6–43.1)	34.6 (25.6–44.9)	41.6 (33.5–53.5)	49.9 (32.9–52.9)	**0.003** *
Asthma Duration in years	19.8±8.9	23.7±12.4	23.3±12.9	27.8±12.5	**0.034** *
Baseline FEV_1%predicted	80.9 (70.5–88.5)	87.1 (79.4–94.5)	85 (57.3–91)	70 (54.3–81.6)	**<0.001** *, †
Baseline FVC%predicted	90.9±15.5	98.2±9.6	87.1±17.7	84.3±17.1	**0.004** †
FEV_1/FVC	0.73 (0.68–0.78)	0.72 (0.70–0.73)	0.72 (0.65–0.80)	0.70 (0.69–0.73)	0.457
Maximum FEV_1%predicted	89 (81.5–99)	101 (90.8–109)	97 (78–106)	83 (67–93)	**<0.001** †, ǁ
Maximum FVC%predicted	97±15	104±10	97±14	92±17	**0.021** †
Maximum FEV_1/FVC %pred	0.84 (0.80–0.86)	0.97 (0.94–1.01)	0.81 (0.79–0.85)	0.88 (0.81–0.95)	**<0.001** †,‡,ǁ
Maximum Reversal to albuterol	11.7 (7–20)	9.7 (5–16)	12.2 (9–30)	12.4 (7–18)	0.356
BALF Yield (return %)	0.56±0.13	0.44±0.14	0.43±0.12	0.37±0.14	**<0.001** *,ǁ,**
BAL Total cell count	9.7 (5.7–18.1)	3.2 (1.9–5.4)	8.6 (6.6–16.9)	4.5 (2.8–6.8)	**<0.001** *,‡,§,ǁ
BALF Macrophage/Monocyte %	92 (87–95)	92 (89–96)	92 (79–95)	91 (83–94)	0.431
BALF Lymphocyte %	5.9 (2.9–10.9)	4 (2.5–8)	3.7 (2.9–15)	5 (3.1–10.3)	0.533
BALF Neutrophil %	1.3 (0.55–2)	1.25 (0.4–2.4)	1.3 (0.2–3.3)	1.8 (0.5–4)	0.710
BALF Eosinophil %	0.3 (0–1)	0.25 (0–1.15)	0.6 (0–0.9)	0.3 (0–1)	0.992
Inhaled Corticosteroid (%positive) in past yr	58.8	80.0	100	100	**<0.001**
Daily Oral Corticosteroid use (%positive)	2.38	0	0	21.62	**0.002**
Long Acting Beta-Agonist use (%positive) in past yr	52.1	35.7	90.9	94.6	**<0.001**
Leukotriene Receptor Antagonist (%positive)	14.6	10.7	45.4	35.1	**0.014**

Pairwise comparisons by Dunn's method post-hoc tests of continuous variables with significance; p values were <0.05 for each:

*for severe PC1negative vs nonsevere PC1 positive

†for severe PC1negative vs nonsevere PC1negative

‡for nonsevere PC1negative vs severe PC1positive

§for severe PC1positive vs severe PC1 negative

// for nonsevere PC1 negative vs nonsevere PC1 positive

** for nonsevere PC1 positive vs severe PC1 positive.